Staphylococcal nuclease and its complexes with nucleotidic inhibitors

A photo-CIDNP study of aromatic residues exposure

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Received 21 February 1983

A laser photo-CIDNP study at 360 MHz of Staphylococcal nuclease shows that calcium binding induces a switch in the accessibility of Tyr_{85} and Tyr_{115}. This switch, which can be reverted by subsequent addition of pdTp inhibitor, might be relevant to the induction of nucleolytic activity upon calcium binding. The conformations of the ternary complexes of calcium (containing nuclease with pdTp, $dTpCH_2dTp$ and $dTpCH_2dTpCH_2dT$) are very similar and are interpreted in terms of comparable binding patterns at the active site of the enzyme.

Staphylococcal nuclease *NMR* *Photo-CIDNP* *Phosphonates* *Interaction*

1. INTRODUCTION

Staphylococcal nuclease (ribonucleate (deoxyribo-nucleate) 3′-nucleotidohydrolase; EC 3.1.4.7.) is a calcium-activated extracellular phosphodiesterase produced by several strains of *Staphylococcus aureus*. It degrades both DNA and RNA to 3′-nucleotides. The enzyme has a single chain of 149 amino acid residues with no disulfide bridges. Although nuclease has been extensively used for elucidating important properties associated with enzyme function and protein structure and folding (review [1–4]) the details of its catalytic mechanism remain unclear. A mechanism of action has been proposed based on the analysis of the structure of a nuclease-thymidine 3′, 5′-biphosphate–Ca^{2+} complex which has been determined by X-ray crystallography at 1.5 Å resolution [5]. On the basis of this study and previous NMR analysis [6,7], several tyrosine residues have been assigned a critical role in the nucleotide and calcium binding processes.

The use of laser photo-chemically induced dynamic nuclear polarization (CIDNP) has been introduced [8] for probing exposure and interaction of aromatic residues in proteins. In this technique, nuclear spin polarization is generated in a number of aromatic residues by reversible electron transfer or hydrogen abstraction reactions with a photoexcited flavine dye which is excited by argon laser irradiation directly in a 360 MHz NMR probe. Tyrosine, histidine and tryptophan residues can be specifically polarized provided they are accessible to the dye: they then appear as positively (Trp, His) or negatively (Tyr) enhanced lines in the aromatic region of the proton NMR spectrum.

Using this technique, we have obtained additional information on the exposure of aromatic residues in native staphylococcal nuclease in solution. In an attempt to determine the conformational changes involved in the binding of calcium and nucleotidic inhibitors, complexes of nuclease with calcium and pdTp have also been studied. To mimic more closely the structure of an en-

Published by Elsevier Biomedical Press B.V.
00145793/83/$3.00

zyme–substrate complex, several complexes of the nuclease with dinucleoside and trinucleoside phosphonate analogs have been studied and the perturbations of the photo-CIDNP effects have been interpreted in terms of conformational rearrangements.

2. MATERIALS AND METHODS

Staphylococcal nuclease was prepared by the procedure in [9] as modified in [10]. Stability of nuclease preparations is greatly enhanced by using in the last step of the purification a chromatography on phosphocellulose with ammonium acetate at pH 6.0 as elution buffer. Deoxythymidine 3′,5′-biphosphate (pdTp) was purchased from Sigma. Di- and trinucleoside phosphonate analogs where chemically synthesized at the Syntex Institute of Molecular Biology (Los Altos CA). Inspection of the nuclease three-dimensional structure was conducted on a graphics display system (Evans and Sutherland PS 2 model) located at the Centre de Recherche sur les Mécanismes de la Croissance Cristalline (Marseille). ^{1}H photo-CIDNP spectra were obtained at 360 MHz using a Bruker HX-360 NMR spectrometer (details in [8]). A Spectra Physics Model 171 argon ion laser was used as the light source and the light was coupled into the NMR probe using an optical fibre. Free induction decays were obtained alternatingly for light and dark conditions and subtracted to give the photo-CIDNP difference spectrum. A 0.6 s (5 W) light pulse was used with a 14 s cycle time. *N*-Carboxymethyllumiflavin was used as the dye at 0.4 mM. All chemical shifts are expressed with respect to DSS.

3. RESULTS

3.1. *pH dependence of the photo-CIDNP difference spectra of Staphylococcal nuclease*

Out of the 7 tyrosine residues of staphylococcal nuclease, only 2 give rise to a photo-CIDNP effect in presence of calcium. A strong emission line centered at 6.91 ppm (Tyr A) and a weaker polarization at 6.84 ppm (Tyr B) are observed for the orthoprotons of 2 tyrosine residues (fig. 1b) at pH 5.15. The usual much smaller accompanying effects on the C-2,6 metaprotons are visible at 7.07 and 7.15 ppm. These sets of signals remain essentially unchanged in position and intensity when pH is increased to 8.8 (fig. 1).

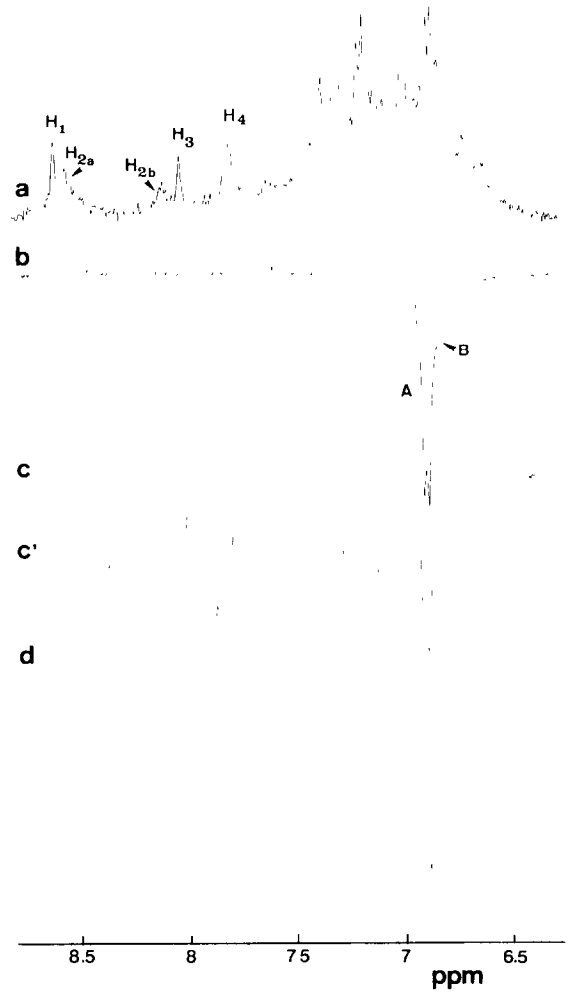

Fig. 1. pH dependence of the 360 MHz proton photo-CIDNP difference spectrum of the aromatic region of Staphylococcal nuclease: (a) dark spectrum 0.9 mM nuclease, 2 mM Ca^{2+} (pH 5.05), 250 scans; (b) difference spectrum (pH 5.15), 10 scans; (c) difference spectrum (pH 6.50), 10 scans; (c′) vertical expansion of (c); (d) difference spectrum at pH 8.80, 10 scans.

Two histidine residues (out of the 4 present in the enzyme) are polarized in presence of the photo-excited dye. The difference spectra at pH 5.15 (fig. 1b) display two sets of emission lines corresponding to the C-2 protons (7.85 and 7.82 ppm) and the C-4 protons (7.37 and 7.24 ppm) of two accessible histidines. According to the numbering generally used for the His residues of Staphylococcal

nuclease [11,12], the low field C-2 proton corresponds to H_{2a} and the higher field C-2 proton to H_3. As described [12] the observation of two signals H_{2a} and H_{2b} corresponding to one single C-2 proton of a histidine residue reflects the existence of a highly localized slow conformational transition between two states (E) and (E'). This transition affects specifically the environment of histidine residue H_2. The polarization of H_{2a} and H_3 signals is enhanced at higher pH indicating that the corresponding His residues become increasingly exposed with deprotonation of the imidazole ring. This effect is particularly marked on the H_{2a} resonance.

At all pH values, no polarization is observed for the unique tryptophan at position 140, indicating a buried location in the three-dimensional structure for this residue.

3.2 *Effect of calcium and inhibitor binding*

At pH 8.6, the binding of Ca^{2+} to Staphylococcal nuclease induces a significant alteration of tyrosine exposure as indicated by pronounced spectral changes (fig. 2). In the absence of calcium (fig. 2a), two emission lines assigned to the C-3,5 protons of 2 tyrosine residues (Tyr A and Tyr C) are observed. When calcium is added (fig. 2b), Tyr A remains unaffected while Tyr C is suppressed. A shoulder (Tyr B) appears on the high field side of the Tyr A signal. Thus, calcium binding clearly unveils a new Tyr residue while masking another one. The alternative explanation that a line is shifting to lower field in the presence of calcium can be confidently excluded, based on previous extensive proton NMR studies [6,14] which unambiguously showed that no tyrosine signal shifts occur upon Ca^{2+} binding. Tyr B and Tyr C resonances correspond then to two different residues and do not reflect different positions of the same tyrosine signal.

The addition of pdTp to nuclease in absence of calcium (fig. 2c) does not modify very significantly the photo-CIDNP effect. The only observed perturbation is a slight upfield shift of the Tyr C signal. By contrast, the addition of pdTp to the calcium-nuclease binary complex (fig. 2d) restores the photo-CIDNP effect on Tyr C and suppresses Tyr B polarization whereas Tyr A remains unaffected. In all cases, the two histidines display the same exposure.

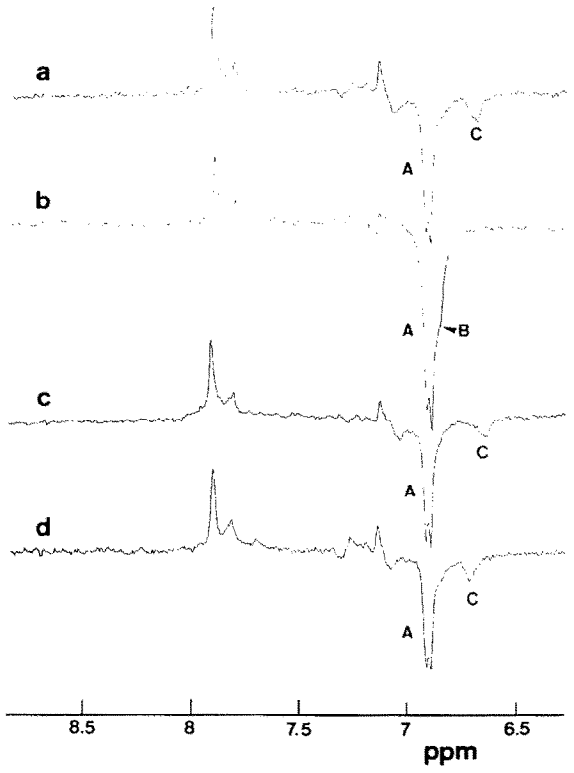

Fig. 2. Effect of calcium and pdTp on the aromatic region of the 360 MHz proton photo-CIDNP difference spectrum of Staphylococcal nuclease at 0.9 mM: (a) pH 8.6 without Ca^{2+}; (b) pH 8.80, Ca^{2+} 2 mM; (c) pH 9.0 without Ca^{2+}, 2 mM pdTp; (d) pH 8.05, 2 mM Ca^{2+}, 2 mM pdTp.

3.3. *Binding of di- and trinucleotide analogs*

Di- and trinucleoside phosphonate analogs that possess a nuclease-resistant phosphonyl (3'→5') linkage (e.g., dTp-CH_2-dTp and dTp-CH_2-dTp-CH_2-dT) in which the 5'-oxygen has been replaced by a methylene group, are strong inhibitors of Staphylococcal nuclease. Solution and crystallographic studies of their binding properties have provided a plausible map of the three-dimensional limits of the nuclease active site [2].

The photo-CIDNP effect on His and Tyr residues remains almost identical when mono-, di- and trinucleotide inhibitors interact with the nuclease (fig. 3). The only difference is a slight shift of Tyr C signal which moves to higher field as the length of the inhibitors increases.

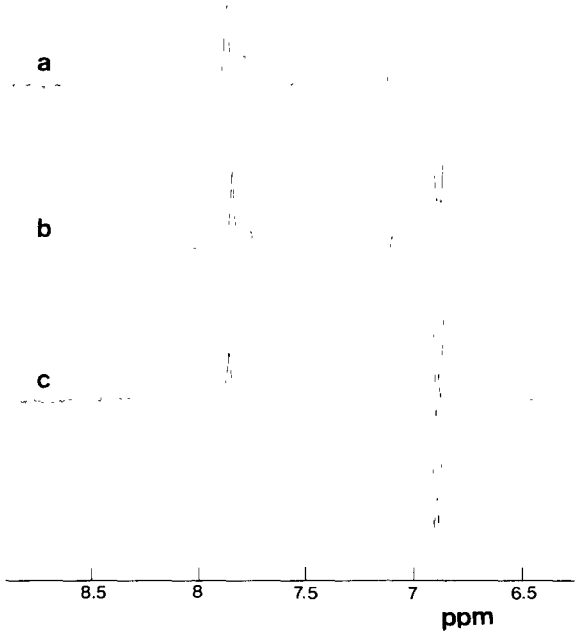

Fig. 3. 360 MHz proton photo-CIDNP difference spectrum of the aromatic region of Staphylococcal nuclease complexes with nucleotidic inhibitors: 0.9 mM nuclease; 2 mM calcium; (a) pH 8.05 with 2 mM pdTp; (b) pH 8.80 with 1.8 mM dTp-CH_2-pdTp; (c) pH 8.9 with 1.3 mM dTp-CH_2-pdTp-CH_2-pdT.

4. DISCUSSION

4.1. *Histidine resonances*

The strongly polarized histidine signal corresponds to the line assigned by [14] to the C-2 proton of His 46. Indeed, the X-ray model of Staphylococcal nuclease shows that His 46 is a very exposed surface residue. Previous NMR work [6,7,12] has shown that this C-2 proton give rise to 2 signals, H2a and H2b (fig. 1a) which reflect 2 slowly exchanging conformational states in the vicinity of His 46. The photo-CIDNP effect is observed in only one of the two conformations (E conformation). Since the conformational fluctuation is known to be highly localized, this very specific photo-CIDNP effect suggests that it reflects the imidazole ring of His 46 swinging in and out of a cleft region.

The weakly polarized H_3 resonance corresponds to the C-2 proton of His 124 as assigned in [13].

Inspection of the X-ray structure of Staphylococcal nuclease shows that His 46 is the most exposed of the 4 histidine residues. It is located at the surface of the protein, with no other atoms within 4 Å and only the sidechains of Lys 48 and Lys 49 lying between 4 and 5 Å. Residue His 124 is slightly less accessible with the sidechain of Glu 101 found at about 4 Å from the imidazole ring. The imidazole nitrogens of His 8 and His 121 are hydrogen-bonded to the peptidic N-H at Lys 9 and the carboxylate group of Glu 75, respectively. These hydrogen bonds probably inhibit the photoreaction with the dye [8].

The X-ray structure can then account for the observation of only 2 histidine residues in the photo-CIDNP experiment and the corresponding assignment of the 2 polarized residues to His 46 and His 124 is in agreement with previous NMR work [14].

The comparison of histidine polarization in fig. 2 and 3 shows that the accessibility of His residues is essentially unaffected by the presence of calcium, pdTp or di- and trinucleoside phosphonate inhibitors.

4.2. *Tyrosine resonances and calcium effect*

Assignment of tyrosine polarization to specific residues cannot be made with absolute certainty at this point. However, reasonable tentative assignments can be proposed based on:

(i) The accessibility of the tyrosine sidechains as deduced from X-ray structure inspection;
(ii) Their known sensitivity toward chemical modification in presence and absence of pdTp [2,3];
(iii) Previous NMR work [6,7,14].

Tyrosine residues at positions 27, 91 and 93 are buried, and their phenolic-OH groups are involved in hydrogen bonding. Therefore, it is very unlikely that a photo-CIDNP effect could be observed for these 3 residues. Out of the remaining 4 tyrosines, the residue at position 54 has a fully exposed surface sidechain with no residue lying within 5 Å. Tyr 85, Tyr 113 and Tyr 115 are found in the cleft region which is known to be the nucleotide binding site [5]. Their hydroxyl groups display various degrees of accessibility.

The strongest tyrosine polarization at 6.91 ppm (Tyr A), which is present in all spectra, probably belongs to Tyr 54. It is the most accessible residue and it is not affected by ligand binding. The phenolic -OH group of Tyr 85 is known to be

hydrogen-bonded to the 3′-phosphate of pdTp [5,7]; in addition, this residue is easily nitrated in the absence of inhibitor but unreactive in the inhibited nuclease [3]. Therefore, it is very likely candidate for Tyr B whose polarization disappears upon addition of pdTp (fig. 2).

An opposite reactivity has been noted for Tyr 115 which is unreactive in the free form and becomes sensitive to nitration and exposed to the solvent in presence of pdTp [3]. This parallels the behavior of Tyr C (fig. 2) which can then be most likely identified as Tyr 115 although an alternative assignment to Tyr 113 cannot be totally excluded at present. The exposure of Tyr C (Tyr 115) in the solution structure of Staphylococcal nuclease is strikingly consistent with the crystallographic results [3] which suggested that upon pdTp binding, Tyr 115 swings out of its pocket region to lie roughly parallel to the pyrimidine ring of the inhibitor. In the uninhibited enzyme, Tyr 115 is folded into the pocket area which delineates the nucleotide binding site.

Interestingly, comparison of spectra a and b in fig. 2 shows that Tyr C is already accessible in the calcium-free enzyme and is suppressed in presence of calcium. It goes back to the surface when pdTp is added (fig. 2d). This observation suggests that calcium binding induces a conformation change which had not been detected before since it does not alter chemical shifts. While masking Tyr C (Tyr 115), this calcium-induced rearrangement unveils Tyr B (Tyr 85).

The chemical shift of Tyr C (fig. 3) is mildly sensitive to the nature of nucleotide in the ternary complex.

5. CONCLUSIONS

This series of experiments clearly shows that Ca^{2+} is able to induce a switch in the accessibility of Tyr B (Tyr 85) and Tyr C (Tyr 115) of Staphylococcal nuclease. This switch, which had not been identified previously, can be reverted by subsequent addition of nucleotide to the enzyme. This conformational transition may be particularly relevant to nuclease activity since calcium-free nuclease is devoid of nucleolytic properties. The photo-CIDNP effect of the histidine residues provides a direct confirmation of the existence of 2 slow-exchanging conformational states, (E) and (E′), in the vicinity of His 46 of Staphylococcal nuclease. Moreover, the observation that the imidazole ring of His 46 becomes exposed only in the conformational state (E) provides a good illustration of the validity and usefulness of the photo-CIDNP experiment to probe the discrete conformational fluctuations which might occur on a protein. Finally, the conformations of the ternary complexes of calcium-containing nuclease with pdTp, $dTpCH_2dTp$ and $dTpCH_2dTpCH_2dT$ are very similar as far as the exposure of His and Tyr residues is concerned. Slight differences are only expressed in the variation of the chemical shift of the Tyr C metaprotons and confirm very similar binding patterns of mono-, di- and trinucleosides to the active site of Staphylococcal nuclease.

ACKNOWLEDGEMENTS

The authors thank Professor O. Jardetzky (Stanford University) for his interest in the early stage of this project and Drs J. Mofatt and G. Jones (Syntex Corporation, Los Altos, CA) for their invaluable help in synthesizing nucleoside phosphonates inhibitors. The assistance of G. Pèpe (CRMC2, Marseille) in the initial use of the graphics display system was appreciated. The award of a FEBS fellowship to complete this research is gratefully acknowledged. This work is supported by grants from CNRS (LA 202), DGRST (MRM 80.7.0151 and TLBM 81-M-0914), INSERM (CRL 813006) and the Fondation pour la Recherche Médicale. The experiments carried out at the Dutch National NMR facility in Groningen were supported by the Netherlands Foundation for Chemical Research (SON) and the Organization for the Advancement of Pure Research (ZWO).

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